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ORIGINAL ARTICLE

Immunocytes modulate ganglionic nitric oxide release which later affects their activity level

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Abstract

Pedal ganglia excised and maintained in culture for up to 2 h, release NO at low levels. The range can vary between 0 to 1.1 nM. Non-stimulated immunocytes do not significantly stimulate ganglionic NO release when incubated with pedal ganglia. However, ganglia exposed to immunocytes that had been previously activated by a 30 min incubation with interleukein1β, release NO significantly above basal levels. In these experiments, 91 ± 2.5% of the non-stimulated immunocytes exhibited form factors in the 0.72 to 0.89 range (sampled prior to ganglionic addition), whereas 62 ± 10.3% of the interleukin 1β stimulated immunocytes had form factors in the 0.39 to 0.49 range, demonstrating activation. Addition of the nitric oxide synthase inhibitor, L-NAME (10^{-4} M), inhibited basal ganglionic NO release as well as that initiated by exposing the ganglia to activated immunocytes. Interestingly, non activated immunocytes, following ganglionic exposure, exhibited activity levels in the 13% range, representing a non significant increase. Cells exposed to interleukin 1β had a 65% activity level at the beginning of the experiment, followed by a drop of activity to 19 ± 3.2% after ganglionic exposure. Repeating this last observation in the presence of L-NAME (10^{-4} M), brought the activity level of the immunocytes back to the pre-ganglionic exposure level of activity, demonstrating that ganglionic NO was involved in down regulating immunocyte activity.

Abbreviations & Units

cNOS	constitutive nitric oxide synthase;
NO	nitric oxide;
ASW	artificial sea water;
ANOVA	analysis of variance;
SEM	standard error of the mean;
FF	form factor;
L-NAME	Nω-nitro-L-arginine methyl ester;
NOS	nitric oxide synthase

Introduction

Mytilus edulis ganglionic tissues contain a population of immunocytes that can freely enter the neural structures via the open circulatory system [1]. In other reports, we demonstrated the presence of microglia associated with neurons, as well as nerve fibers, in the same neural tissues [2,3]. The reports also demonstrate that the ganglionic microglia can become active after ganglionic excision and egress from the tissue through the nerves that are severed. Additionally, we have shown that *Mytilus* neural tissues contain mammalian-like cytokines, suggesting their signaling potential in these tissues [4–9]. The cytokine reports further reveal that mammalian cytokines can activate invertebrate immunocytes, making them amoeboid and motile, as well as secrete cytokines. Recently, it was shown that *Mytilus edulis* possesses constitutive nitric oxide synthase (cNOS) processes in its nervous and immune tissues [10,11]. This complements the reports demonstrating that cNOS-derived nitric oxide has the potential to down regulate immunocyte activity, causing the cells to become round, inactive and non-adherent [3,10–16].

Given the demonstration of the above signaling processes, it was of interest to initiate studies on the effect of immunocytes and ganglionic signaling, which may affect ganglionic functions, demonstrating neuroimmune communication. In the present report, we demonstrate that interleukin 1 (IL-1β) activates immunocytes and by exposing *Mytilus edulis* ganglionic tissues to these cells, nitric oxide release is initiated, which then alters the activity state of the immunocytes. Taken together, invertebrate immune and neural tissues have the ability to communicate with each other.

Material and Methods

Mytilus edulis, a marine bivalve mollusk, were maintained in the laboratory for three weeks prior to their dissection [17]. Invertebrate immunocytes were collected and processed as described elsewhere in detail [18,19]. The pedal ganglia were removed and placed on ice and rinsed in artificial sea water (ASW; Instant Ocean, Inc., Boston, MA) and cell-free (verified by light microscopic inspection) filtered and centrifuged (900 x g for 10 min) hemolymph 1:1 by volume [20,21]. This incubation medium also contained streptomycin (50 mg/100 ml), penicillin (30 mg/100 ml) and gentamycin (50 mg/ml) to reduce any bacterial presence [2]. The excised ganglia (6 per test in 2 ml of the indicated medium x 5 replicates) were incubated for 1 h at room temperature. Pharmacological exposure of the immunocytes to the respective drugs occurred for an additional 30 min followed by a brief washing. At the end of this time period, drug exposed immunocytes (10^6 immunocytes/ in 1 ml) were added to the ganglionic preparations for nitric oxide (NO) detection. After a 30 min delay in which NO release did not occur to any significant extent above background, NO evaluation began.

The NO levels recorded at the indicated times were combined with four other values (preparations) per treatment (±SEM) and compared via a paired ANOVA for repeated measures to vehicle-treated controls; the criterion for significance was $P < 0.05$. Vehicle controls were those containing or involving non-treated tissues.

NO Determination

The respective tissues were bathed in the incubation medium described earlier. NO release was monitored with a NO-selective microprobe manufactured by World Precision Instruments (Sarasota, FL). Tip diameter of the probe (200 μm) permitted the use of a micromanipulator (Zeiss-Eppendorff) attached to the stage of an inverted microscope (Nikon Diaphot) to position the sensor 20 μm above the ganglion surface. The system was calibrated daily by adding potassium nitrite to a solution of potassium iodide, resulting in the liberation of a known quantity of NO. The probe was routinely cleaned by gentle rinsing to remove cellular debris that tends to accumulate on it. The probes used for these experiments are routinely maintained in ASW and attached to the metering system when not in use. The probe is allowed to equilibrate for 30 min in artificial seawater before being transferred to vials containing the tissue for another 30 min. Manipulations/handling of the cells was only performed with glass instruments. Each experiment was repeated 5 times and the NO mean values obtained were graphed to represent the actual NO release (± SEM).

The data obtained was then evaluated by a paired ANOVA for repeated measures. Data acquisition was by the computer interfaced DUO-18 software (World Precision Instruments, Sarasota, FL.). The experimental values were then transferred to Sigma-Plot and -Stat (Jandel, CA) for graphic representation and evaluation. Data gatherers were unaware of the experimental conditions.

Morphological analysis of glia and immunocytes

The activity state of the immunocytes was measured via morphological measurements. This determination is based on cell area and perimeter measurements as measured by image analysis software (Image Analytics, Inc., Hauppauge, NY). Form-factor (FF) calculations were performed as previously described. [22,23]. The observational area used for measurement determinations and frame-grabbing of the respective immunocytes was 400 μm in diameter. The computer-assisted microscopic image analysis system (Zeiss Ax-

iophot fitted with Nomarski and phase contrast optics) was the same as previously described [24]. The cells were analyzed for conformational changes indicative of either activation (amoeboid and mobile) or inhibition (round and stationary). The lower the FF number, the longer the perimeter and the more amoeboid the cellular shape. The proportion of activated cells was determined as previously described [24]. Briefly, with phase-contrast optics round cells appear bright yellow whereas amoeboid cells dark. Based on this color difference, the software is manually taught to differentiate these colors and then count the cells in each group and by comparison, the percent activation can be calculated from the uniform number of cells harvested for examination (400 ± 11/ 0.2 ml). It should also be noted that the activated state (amoeboid conformation) of human and invertebrate cells is correlated to biochemical adhesion molecule alterations [23] as well as cytokine production [4,6].

Results

Pedal ganglia excised and maintained in culture for up to 2 h, release NO at low levels during this period. The range can vary between 0 to 1.1 nM [10,25] (Figure 1). Invertebrate immunocytes, that also release NO [10,11,14,25,26], produce it within the same concentration range [27] (Figure 1). Thus, it was of interest to determine if immunocytes added to cultured ganglia would enhance basal/constitutive ganglionic NO production since immune cells, e.g., microglia and immunocytes, have been found and identified in molluscan ganglia, specifically, *Mytilus* pedal ganglia [1–3,13].

In this regard, non-stimulated immunocytes do not significantly stimulate ganglionic NO release when incubated with pedal ganglia (Figures 1–3). However, ganglia exposed to activated immunocytes (10^6 / ml; [pre-exposed for 30 min to an effective concentration of interleukin 1β, 1 ng] [8] for 30 min before being added to the ganglionic incubation medium), release significant levels of NO above basal levels (Figures 1–3) after 30 min of the cells addition. In these experiments, 91 + 2.5% of the non-stimulated immunocytes exhibited form factors in the 0.72 to 0.89 range (sampled prior to ganglionic addition), whereas 62 ± 10.3% of the interleukin 1β stimulated immunocytes had form factors in the 0.39 to 0.49 range, having an amoeboid shape, as well as being motile (n=5; comparison of the% activated by a one–tailed student's t-test revealed a $P < 0.005$). Addition of the nitric oxide synthase (NOS) inhibitor, L-NAME (10^{-4} M), inhibited basal ganglionic NO release as well as that initiated by exposing the ganglia to activated immunocytes, substantiating the identity of the material being monitored (Figure 2).

Given the above results, it was of interest to determine the activity state of the immunocytes following their incubation with the non-exposed and activated immunocyte exposed ganglia (Figure 3). Non activated immunocytes, following ganglionic exposure, exhibited activity levels in the 13% range, representing a non significant increase (Figure 3 inset). Cells exposed to interleukin 1β had a 65% activity level at the beginning of the experiment, followed by a drop of activity to 19 ± 3.2% after ganglionic exposure. Repeating this last observation in the presence of L-NAME (10^{-4} M), brought the activity level of the immunocytes back to the pre-ganglionic exposure level of activity (Figure 3, inset), demonstrating that ganglionic NO was involved in down regulating immunocyte activity.

Discussion

The present study demonstrates that *Mytilus* immune cells, i.e., immunocytes, have the ability to alter ganglionic processes, i.e., cNOS derived NO release. Additionally, ganglionic NO release can then affect immunocyte activity; and in this instance, it down regulates the active cells. Taken together, these data demonstrate that immune neural signaling occurs in this mollusk.

This current study supports data obtained in previous studies that demonstrate that cNOS derived NO can down regulate *Mytilus* immunocytes, as well as human leukocytes [11,12]. Furthermore, in numerous reports we demonstrate that morphine stimulation is, in part, coupled to NO release, accounting for its immune down regulating properties related to cell and proinflammatory activities [10,15,28–33]. The present report demonstrates that within the behavioral response of the ganglia to a "proinflammatory-type" stimulus, the ganglia cause NO release to counter this physiological state, down regulating the activated immunocytes. Thus, it would appear that a normal function of cNOS derived NO is to limit immune activation [31]. In this regard, we surmise that the basal, unstimulated levels of NO produced by the ganglia may limit microenvironmental noise[27,31,34] by maintaining cells in a mild inhibitory state.

With regard to ganglionic function, *Mytilus* ganglia, among many functions, serve to modulate the organisms lateral gill ciliary (beating rate) and foot activity (extension, contraction and widening as well as the laying down of byssus threads) [35,36,37,38,39]. Dopaminergic transmission results in the contraction of the foot, bringing it into the shell, whereas in the gill, it inhibits the beating of the lateral gill cilia that normally beat and allow water (food, oxygen and waste) to move through. Thus, in a coordinated manner, if dopamine signaling is occurring, the water stops flowing and the foot comes back into the shell environment. In more recent times, we found that NO can inhibit dopamine release from pre-synaptic terminals in the ganglia of *Mytilus* [40]. Given the present findings of activated immunocytes initiating NO release, we surmise that this signaling gas stops dopaminergic signaling, allowing for ciliary activation and foot extension, leading to water flow, which "flushes" the organism's internal tissues. In a natural environment, such a "normal" stimulus may be originated by bacteria.

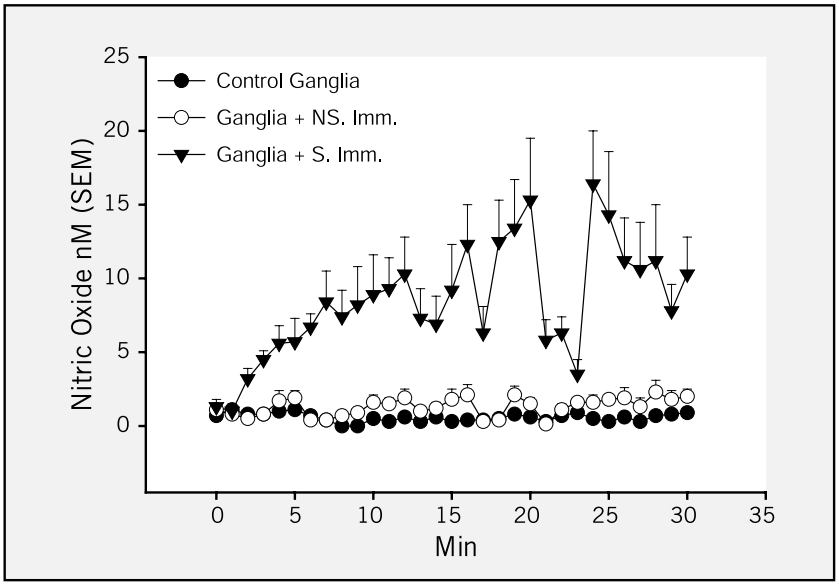

Figure 1. The effect of exposing interleukin 1β stimulated immunocytes (Imm) to excised *Mytilus* pedal ganglia. Thirty minutes following incubation stimulated (S) and non-stimulated (NS) cells with pedal ganglia, the ganglia produce significantly higher nitric oxide levels only in the S exposure. Details of the preparations are found in the text. Each test involved 6 ganglia per treatment, replicated five times.

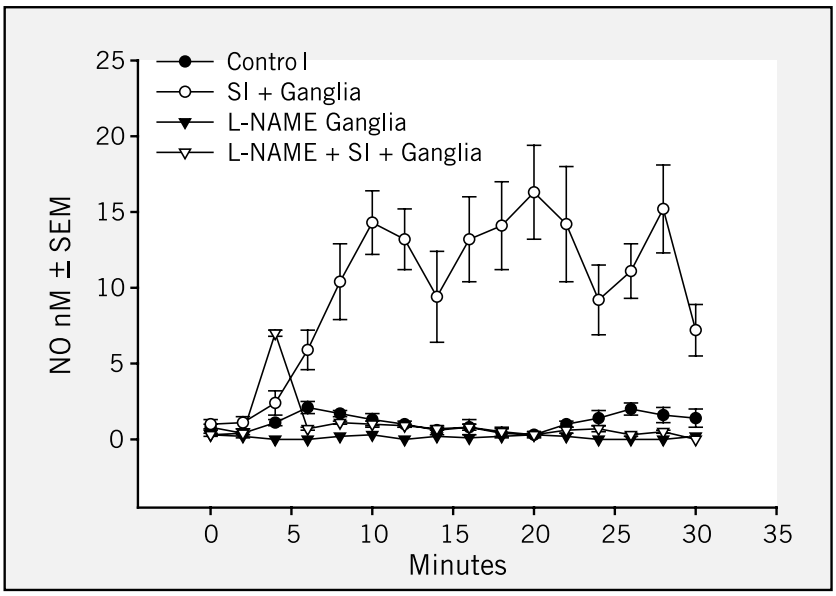

Figure 2. The effect of L-NAME treatment to ganglia exposed to interleukin 1β stimulated immunocytes (SI). Thirty minutes following incubation of stimulated and non-stimulated cells with pedal ganglia, the ganglia produce significantly higher nitric oxide levels only in the stimulated immunocyte (SI) exposure. The addition of L-NAME to the non-SI exposed ganglia resulted in simply lower basal NO ganglionic levels .In the SI scenario, pre-ganglionic exposure to L-NAME suppressed the SI effect. Details of the preparations are found in the text. Each test involved 6 ganglia per treatment, replicated five times.

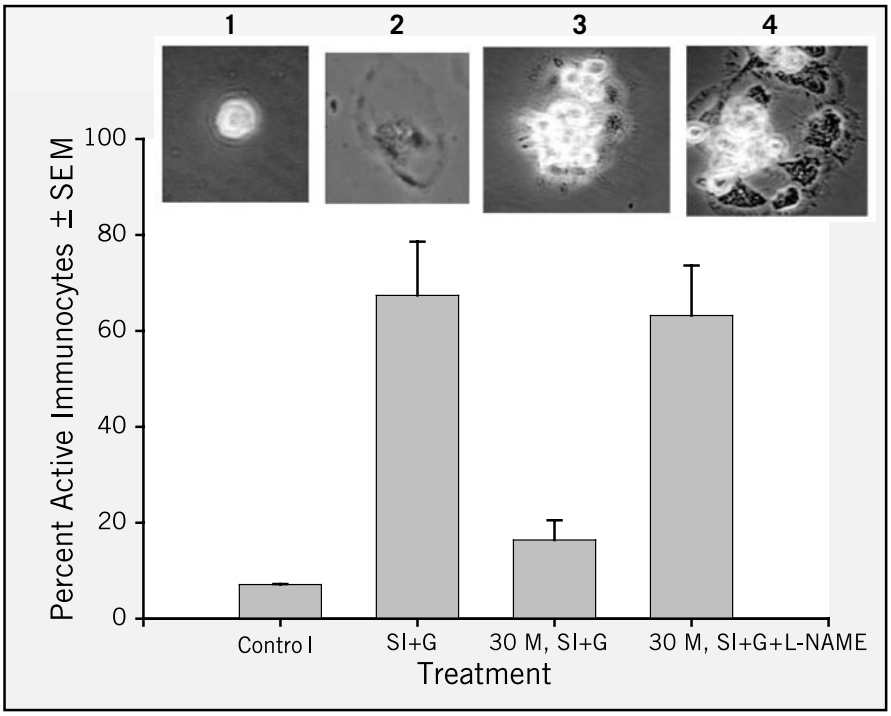

Figure 3. The effect of the various treatments on the activity state of the immunocytes. Control immunocytes exhibit form factors in the 0.79 to 1 range, indicating a round non-motile conformation (see inset 1). Additionally, when first obtained from the animal only 7-9% of the cells are active. Interleukin 1β stimulated immunocytes (SI), in total, have approximately 65% of the cells in an amoeboid shape (inset 2) and are motile. 30 min after exposing the ganglia to SI, only about 18% of the cells remain active (inset 3). Lastly, adding L-NAME to the incubation medium (10-4 M), and repeating the previous experiment, we find that about 63% of the cells are active, exhibiting amoeboid conformations and moving (inset 4). ANOVA analysis revealed statistical significance ($P < 0.01$) in the comparison of control to SI + G and L-NAME exposed preparations and in comparing the L-NAME with the non-L-NAME 30 min cells.

Furthermore, as in the case of recently activated immunocytes, once NO is released and begins to down regulate these same cells, normal activity may resume. Certainly, at the present time, a cascade of possibilities exists. However, it is important to note that these demonstrated responses to excitation all fall within the realm of possibilities discussed due to the fact that these neurological processes are present. This study also reveals that in this particular invertebrate, which has a relatively long life span, neuroimmune communication occurs.

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